α-amylase mutants

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. application no. 09/769,864, filed on January 25, 2001, which is a divisional of U.S. application No. 09/183,412, filed on October 30, 1998, and claims priority under 35 U.S.C. 119 of Danish application no. 1240/97, filed on October 30, 1997, Danish application no. PA 1998 00936, filed on July 14, 1998, U.S. provisional application no. 60/064,662, filed on November 6, 1997 and U.S. provisional application no. 60/093,234, filed on July 17, 1998, the contents of which are fully incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to variants (mutants) of parent Termamyl-like α-amylases with higher activity at medium temperatures and/or high pH.

BACKGROUND OF THE INVENTION

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

There is a very extensive body of patent and scientific literature relating to this industrially very important class of enzymes. A number of α-amylases such as Termamyl-like α-amylases variants are known from *e.g.* WO 90/11352, WO 95/10603, WO 95/26397, WO 96/23873 and WO 96/23874.

Among more recent disclosures relating to α-amylases, WO 96/23874 provides three-dimensional, X-ray crystal structural data for a Termamyl-like α-amylase which consists of the 300 N-terminal amino acid residues of the *B. amyloliquefaciens* α-amylase (BAN™) and amino acids 301-483 of the C-terminal end of the *B. licheniformis* α-amylase comprising the amino acid sequence (the

latter being available commercially under the tradename Termamyl™), and which is thus closely related to the industrially important *Bacillus* α-amylases (which in the present context are embraced within the meaning of the term "Termamyl-like α-amylases", and which include, *inter alia*, the *B. licheniformis*, *B. amyloliquefaciens* (BAN™) and *B. stearothermophilus* (BSG™) α-amylases). WO 96/23874 further describes methodology for designing, on the basis of an analysis of the structure of a parent Termamyl-like α-amylase, variants of the parent Termamyl-like α-amylase which exhibit altered properties relative to the parent.

BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to novel α-amylolytic variants(mutants) of a Termamyl-like α-amylase which exhibit improved wash performance (relative to the parent α-amaylase) at high pH and at a medium temperature.

The term "medium temperature" means in the context of the invention a temperature from 10°C to 60°C, preferably 20°C to 50°C, especially 30-40°C.

The term "high pH" means the alkaline pH which today are used for washing, more specifically from about pH 8 to 10.5.

In the context of the invention a "low temperature α-amylase" means an α-amylase which has an relative optimum activity in the temperature range from 0-30°C.

In the context of the invention a "medium temperature α-amylase" means an α-amylase which has an optimum activity in the temperature range from 30-60°C. For instance, SP690 and SP722 α-amaylases, respectively, are "medium temperature α-amylases.

In the context of the invention a "high temperature α-amylase" is an α-amylase having the optimum activity in the temperature range from 60-110°C. For instance, Termamyl is a "high temperature

α-amylase.

Alterations in properties which may be achieved in variants(mutants) of the invention are alterations in:

the stability of the Termamyl-like α-amylase at a pH from 8 to 10.5, and/or the Ca^{2+} stability at pH 8 to 10.5, and/or

the specific activity at temperatures from 10 to 60°C, preferably 20-50°C, especially 30-40°C.

It should be noted that the relative temperature optimum often is dependent on the specific pH used. In other words the relative temperature optimum determined at, e.g., pH 8 may be substantially different from the relative temperature optimum determined at, e.g., pH 10.

The temperature's influence on the enzymatic activity

The dynamics in the active site and surroundings are dependent on the temperature and the amino acid composition and of strong importance for the relative temperature optimum of an enzyme. By comparing the dynamics of medium and high temperature α-amylases, regions of importance for the function of high temperature α-amylases at medium temperatures can be determined. The temperature activity profile of the SP722 α-amaylase (SEQ ID NO: 2) and the *B. licheniformis* α-amylase (available from Novo Nordisk as Termamyl®) (SEQ ID NO: 4) are shown in Figure 2.

The relative temperature optimum of SP722 in absolute activities are shown to be higher at medium range temperatures (30-60°C) than the homologous *B. licheniformis* α-amylase, which have an optimum activity around 60-100°C. The profiles are mainly dependent on the temperature stability and the dynamics of the active site residues and their surroundings. Further, the activity profiles are dependent on the pH used and the pKa of the active site residues.

In the first aspect the invention relates to a variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity, said variant comprises one or more mutations

corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 2:

T141, K142, F143, D144, F145, P146, G147, R148, G149,
Q174, R181, G182, D183, G184, K185, A186, W189, S193, N195,
H107, K108, G109,D166, W167, D168, Q169, S170, R171, Q172, F173,
F267, W268, K269, N270, D271, L272, G273, A274, L275, K311, E346,
K385, G456, N457, K458, P459, G460, T461, V462, T463.

A variant of the invention have one or more of the following substitutions or deletions:

T141A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
K142A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
F143A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
D144A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F145A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
P146A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G147A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R148A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G149A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R181*,A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G182*,A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G184*,A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K185A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W189A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
S193A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
H107A,D,R,N,C,E,Q,G,I,L,K,M,F,P,S,T,W,Y,V;
K108A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G109A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D166A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W167A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
D168A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q169A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S170A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
R171A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

Q172A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F173A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
Q174*,A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F267A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
W268A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D271A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L272A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G273A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
A274D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L275A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
K311A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
E346A,D,R,N,C,Q,G,H,I,K,L,M,F,P,S,T,W,Y,V;
K385A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G456A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
N457A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G460A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
V462A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
T463A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

Preferred are variants having one or more of the following substitutions or deletions:
K142R; S193P; N195F; K269R,Q; N270Y,R,D; K311R; E346Q; K385R; K458R; P459T; T461P; Q174*; R181Q,N,S; G182T,S,N; D183*; G184*; K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V,R; W189T,S,N,Q.

Especially preferred are variants having a deletion in positions D183 and G184 and further one or more of the following substitutions or deletions:
K142R; S193P; N195F; K269R,Q; N270Y,R,D; K311R; E346Q; K385R; K458R; P459T; T461P; Q174*; R181Q,N,S; G182T,S,N; K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V,R; W189T,S,N,Q.

The variants of the invention mentioned above exhibits an alteration in at least one of the following properties relative to the parent α-amylase:

i) improved pH stability at a pH from 8 to 10.5; and/or

ii) improved Ca^{2+} stability at pH 8 to 10.5, and/or

iii) increased specific activity at temperatures from 10 to 60°C, preferably 20-50°C, especially 30-40°C. Further, details will be described below.

The invention further relates to DNA constructs encoding variants of the invention; to methods for preparing variants of the invention; and to the use of variants of the invention, alone or in combination with other enzymes, in various industrial products or processes, e.g., in detergents or for starch liquefaction.

In a final aspect the invention relates to a method of providing α-amylases with altered pH optimum, and/or altered temperature optimum, and/or improved stability.

Nomenclature

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are used. For ease of reference, α-amylase variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of alanine for asparagine in position 30 is shown as:

Ala30Asn or A30N

a deletion of alanine in the same position is shown as:

Ala30* or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala30AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or Δ(A30-N33).

Where a specific α-amylase contains a "deletion" in comparison with other α-amylases and an insertion is made in such a position this is indicated as:

*36Asp or *36D

for insertion of an aspartic acid in position 36

Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively.

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of:

R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is an alignment of the amino acid sequences of six parent Termamyl-like α-amylases. The numbers on the extreme left designate the respective amino acid sequences as follows:

1: SEQ ID NO: 2

2: Kaoamyl

3: SEQ ID NO: 1

4: SEQ ID NO: 5

5: SEQ ID NO: 4

6: SEQ ID NO: 3.

Figure 2 shows the temperature activity profile of SP722 (SEQ ID NO: 2) (at pH 9) and *B. licheniformis* α-amylase (SEQ ID NO: 4)

(at pH 7.3).

Figure 3 shows the temperature profile for SP690 (SEQ ID NO: 1), SP722 (SEQ ID NO: 2), *B. licheniformis* α-amylase (SEQ ID NO: 4) at pH 10.

Figure 4 is an alignment of the amino acid sequences of five α-amylases. The numbers on the extreme left designate the respective amino acid sequences as follows:

1: amyp_mouse

2: amyp_rat

3: amyp_pig porcine pancreatic alpha-amylase (PPA)

4: amyp_human

5: amy_altha *A. haloplanctis* alpha-amylase (AHA)

DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α-amylase

It is well known that a number of α-amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID NO:. 4 (commercially available as Termamyl™) has been found to be about 89% homologous with the *B. amyloliquefaciens* α-amylase comprising the amino acid sequence shown in SEQ ID NO: 5 and about 79% homologous with the *B. stearothermophilus* α-amylase comprising the amino acid sequence shown in SEQ ID NO: 3. Further homologous α-amylases include an α-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the α-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31, (see SEQ ID NO: 6).

Still further homologous α-amylases include the α-amylase produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the α-amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis*

α-amylases are comprised in the products Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ and Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

Because of the substantial homology found between these α-amylases, they are considered to belong to the same class of α-amylases, namely the class of "Termamyl-like α-amylases".

Accordingly, in the present context, the term "Termamyl-like α-amylase" is intended to indicate an α-amylase which, at the amino acid level, exhibits a substantial homology to Termamyl™, i.e., the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO:4 herein. In other words, all the following α-amylases which has the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 or 8 herein, or the amino acid sequence shown in SEQ ID NO: 1 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 7 herein) or in SEQ ID NO: 2 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 8 herein) or in Tsukamoto et al., 1988, (which amino acid sequence is shown in SEQ ID NO: 6 herein) are considered to be "Termamyl-like α-amylase". Other Termamyl-like α-amylases are α-amylases i) which displays at least 60%, such as at least 70%, e.g., at least 75%, or at least 80%, e.g., at least 85%, at least 90% or at least 95% homology with at least one of said amino acid sequences shown in SEQ ID NOS: 1-8 and/or ii) displays immunological cross-reactivity with an antibody raised against at least one of said α-amylases, and/or iii) is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the above-specified α-amylases which are apparent from SEQ ID NOS: 9, 10, 11, or 12 of the present application (which encoding sequences encode the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4 and 5 herein, respectively), from SEQ ID NO: 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA, is shown in SEQ ID NO: 13 herein and encodes the amino acid sequence shown in

SEQ ID NO: 8 herein) and from SEQ ID NO: 5 of WO 95/26397 (shown in SEQ ID NO: 14 herein), respectively.

In connection with property i), the "homology" may be determined by use of any conventional algorithm, preferably by use of the GAP progamme from the GCG package version 7.3 (June 1993) using default values for GAP penalties, which is a GAP creation penalty of 3.0 and GAP extension penalty of 0.1, (Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711).

A structural alignment between Termamyl (SEQ ID NO: 4) and a Termamyl-like α-amylase may be used to identify equivalent/corresponding positions in other Termamyl-like α-amylases. One method of obtaining said structural alignment is to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., (1987), FEBS LETTERS 224, pp. 149-155) and reverse threading (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998).

Property ii) of the α-amylase, i.e., the immunological cross reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-like α-amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g., as described by Hudson et al., Practical Immunology, Third edition (1989), Blackwell Scientific Publications. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g., as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α-amylases having the amino acid sequences SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, or 8, respectively, has been found.

The oligonucleotide probe used in the characterisation of the Termamyl-like α-amylase in accordance with property iii) above may

suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α-amylase in question.

Suitable conditions for testing hybridisation involve pre-soaking in 5xSSC and prehybridizing for 1 hour at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50mg of denatured sonicated calf thymus DNA, followed by hybridisation in the same solution supplemented with 100mM ATP for 18 hours at ~40°C, followed by three times washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at ~75°C (very high stringency). More details about the hybridisation method can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

In the present context, "derived from" is intended not only to indicate an α-amylase produced or producible by a strain of the organism in question, but also an α-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an α-amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α-amylase in question. The term is also intended to indicate that the parent α-amylase may be a variant of a naturally occurring α-amylase, i.e. a variant which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring α-amylase.

Parent hybrid α-amylases

The parent α-amylase (i.e., backbone α-amylase) may be a hybrid α-amylase, i.e., an α-amylase which comprises a combination of partial amino acid sequences derived from at least two α-amylases.

The parent hybrid α-amylase may be one which on the basis of amino acid homology and/or immunological cross-reactivity and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α-amylase family. In this case, the hybrid α-amylase is typically composed of at least one part of a Termamyl-like α-amylase and part(s) of one or more other α-amylases selected from Termamyl-like α-amylases or non-Termamyl-like α-amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α-amylase may comprise a combination of partial amino acid sequences deriving from at least two Termamyl-like α-amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial α-amylase, or from at least one Termamyl-like and at least one fungal α-amylase. The Termamyl-like α-amylase from which a partial amino acid sequence derives may, *e.g.*, be any of those specific Termamyl-like α-amylase referred to herein.

For instance, the parent α-amylase may comprise a C-terminal part of an α-amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an α-amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*. For instance, the parent α-amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* α-amylase, and may, *e.g.*, comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the *B. amyloliquefaciens* α-amylase having the amino acid sequence shown in SEQ ID NO: 5 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO: 4, or a hybrid Termamyl-like α-amylase being identical to the Termamyl sequence, i.e., the *Bacillus licheniformis* α-amylase shown in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues

(of the mature protein) has been replaced by the N-terminal 33 residues of BAN (mature protein), i.e., the *Bacillus amyloliquefaciens* α-amylase shown in SEQ ID NO: 5; or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearothermophilus* α-amylase having the amino acid sequence shown in SEQ ID NO: 3 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO: 4.

Another suitable parent hybrid α-amylase is the one previously described in WO 96/23874 (from Novo Nordisk) constituting the N-terminus of BAN, *Bacillus amyloliquefaciens* α-amylase (amino acids 1-300 of the mature protein) and the C-terminus from Termamyl (amino acids 301-483 of the mature protein). Increased activity was achieved by substituting one or more of the following positions of the above hybrid α-amylase (BAN:1-300/Termamyl:301-483): Q360, F290, and N102. Particularly interesting substitutions are one or more of the following substitutions: Q360E,D; F290A,C,D,E,G,H,I,K,L,M,N,P,Q,R,S,T; N102D,E;

The corresponding positions in the SP722 α-amylase shown in SEQ ID NO: 2 are one or more of: S365, Y295, N106. Corresponding substitutions of particular interest in said α-amylase shown in SEQ ID NO: 2 are one or more of: S365D,E; Y295 A,C,D,E,G,H,I,K,L,M,N,P,Q,R,S,T; and N106D,E.

The corresponding positions in the SP690 α-amylase shown in SEQ ID NO: 1 are one or more of: S365, Y295, N106. The corresponding substitutions of particular interest are one or more of: S365D,E; Y295 A,C,D,E,G,H,I,K,L,M,N,P,Q,R,S,T; N106D,E.

The above mentioned non-Termamyl-like α-amylase may, e.g., be a fungal α-amylase, a mammalian or a plant α-amylase or a bacterial α-amylase (different from a Termamyl-like α-amylase). Specific examples of such α-amylases include the *Aspergillus oryzae* TAKA α-amylase, the *A. niger* acid α-amylase, the *Bacillus*

subtilis α-amylase, the porcine pancreatic α-amylase and a barley α-amylase. All of these α-amylases have elucidated structures which are markedly different from the structure of a typical Termamyl-like α-amylase as referred to herein.

The fungal α-amylases mentioned above, i.e., derived from *A. niger* and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α-amylases. The fungal α-amylase derived from *Aspergillus oryzae* is commercially available under the tradename Fungamyl™.

Furthermore, when a particular variant of a Termamyl-like α-amylase (variant of the invention) is referred to - in a conventional manner - by reference to modification (e.g., deletion or substitution) of specific amino acid residues in the amino acid sequence of a specific Termamyl-like α-amylase, it is to be understood that variants of another Termamyl-like α-amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

In a preferred embodiment of the invention the α-amylase backbone is derived from *B. licheniformis* (as the parent Termamyl-like α-amylase), e.g., one of those referred to above, such as the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO: 4.

Altered properties of variants of the invention

The following discusses the relationship between mutations which are present in variants of the invention, and desirable alterations in properties (relative to those a parent Termamyl-like α-amylase) which may result therefrom.

Improved stability at pH 8-10.5

In the context of the present invention, mutations (including amino acid substitutions) of importance with respect to achieving

improved stability at high pH (*i.e.*, pH 8-10.5) include mutations corresponding to mutations in one or more of the following positions in SP722 α-amylase (having the amino acid sequence shown in SEQ ID NO: 2): T141, K142, F143, D144, F145, P146, G147, R148, G149, R181, A186, S193, N195, K269, N270, K311, K458, P459, T461.

The variant of the invention have one or more of the following substitutions (using the SEQ ID NO: 2 numbering):

T141A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
K142A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
F143A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
D144A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F145A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
P146A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G147A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R148A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G149A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K181A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,P,K,M,F,S,T,W,Y,V;
S193A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K311A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

Preferred high pH stability variants include one or more of the following substitutions in the SP722 α-amylase (having the amino acid sequence shown in SEQ ID NO: 2):

K142R, R181S, A186T, S193P, N195F, K269R, N270Y, K311R, K458R, P459T and T461P.

In specific embodiments the *Bacillus* strain NCIB 12512 α-amylase having the sequence shown in SEQ ID NO: 1, or the *B. stearothermophilus* α-amylase having the sequence shown in SEQ ID NO: 3, or the *B. licheniformis* α-amylase having the sequence shown

in SEQ ID NO: 4, or the *B. amyloliquefaciens* α-amylase having the sequence shown in SEQ ID NO: 5 is used as the backbone, i.e., parent Termamyl-like α-amylase, for these mutations.

As can been seen from the alignment in Figure 1 the *B. stearothermophilus* α-amylase already has a Tyrosine at position corresponding to N270 in SP722. Further, the *Bacillus* strain NCIB 12512 α-amylase, the *B. stearothermophilus* α-amylase, *the B. licheniformis* α-amylase and the *B. amyloliquefaciens* α-amylase already have Arginine at position corresponding to K458 in SP722. Furthermore, the *B. licheniformis* α-amylase already has a Proline at position corresponding to T461 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

α-amylase variants with improved stability at high pH can be constructed by making substitutions in the regions found using the molecular dynamics simulation mentioned in Example 2. The simulation depicts the region(s) that has a higher flexibility or mobility at high pH (i.e., pH 8-10.5) when compared to medium pH.

By using the structure of any bacterial alpha-amylase with homology (as defined below) to the Termamyl-like α-amylase (BA2), of which the 3D structure is disclosed in Appendix 1 of WO 96/23874 (from Novo Nordisk), it is possible to modelbuild the structure of such alpha-amylase and to subject it to molecular dynamics simulations. The homology of said bacterial α-amylase may be at least 60%, preferably be more than 70%, more preferably more than 80%, most preferably more than 90% homologous to the above mentioned Termamyl-like α-amylase (BA2), measured using the UWGCG GAP program from the GCG package version 7.3 (June 1993) using default values for GAP penalties [Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711]. Substitution of the unfavorable residue for another would be applicable.

Improved Ca^{2+} stability at pH 8-10.5

Improved Ca^{2+} stability means the stability of the enzyme

under Ca^{2+} depletion has been improved. In the context of the present invention, mutations (including amino acid substitutions) of importance with respect to achieving improved Ca^{2+} stability at high pH include mutation or deletion in one or more positions corresponding to the following positions in the SP722 α-amylase having the amino acid sequence shown in SEQ ID NO: 2: R181, G182, D183, G184, K185, A186, W189, N195, N270, E346, K385, K458, P459.

A variant of the invention have one or more of the following substitutions or deletions:

R181*,A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G182*,A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G184*,A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K185A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W189A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
N270A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
E346A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K385A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
K458A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,R,D,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V.

Preferred are variants having one or more of the following substitutions or deletions:

R181Q,N; G182T,S,N; D183*; G184*;
K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V;
W189T,S,N,Q; N195F, N270R,D; E346Q; K385R; K458R; P459T.

In specific embodiments the *Bacillus* strain NCIB 12512 α-amylase having the sequence shown in SEQ ID NO: 1, or the *B. amyloliquefaciens* α-amylase having the sequence shown in SEQ ID NO: 5, or the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4 are used as the backbone for these mutations.

As can been seen from the alignment in Figure 1 the *B. licheniformis* α-amylase does not have the positions corresponding to D183 and G184 in SP722. Therefore for said α-amylases these

deletions are not relevant.

In a preferred embodiment the variant is the *Bacillus* strain NCIB 12512 α-amylase with deletions in D183 and G184 and further one of the following substitutions: R181Q,N and/or G182T,S,N and/or D183*; G184* and/or K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V and/or A186T,S,N,I,V and/or W189T,S,N,Q and/or N195F and/or N270R,D and/or E346Q and/or K385R and/or K458R and/or P459T.

Increased specific activity at medium temperature

In a further aspect of the present invention, important mutations with respect to obtaining variants exhibiting increased specific activity at temperatures from 10-60°C, preferably 20-50°C, especially 30-40°C, include mutations corresponding to one or more of the following positions in the SP722 α-amylase having the amino acid sequence shown in SEQ ID NO: 2:
H107, K108, G109, D166, W167, D168, Q169, S170, R171, Q172, F173, Q174, D183, G184, N195, F267, W268, K269, N270, D271, L272, G273, A274, L275, G456, N457, K458, P459, G460, T461, V462, T463.

The variant of the invention have one or more of the following substitutions:
H107A,D,R,N,C,E,Q,G,I,L,K,M,F,P,S,T,W,Y,V;
K108A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G109A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D166A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W167A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
D168A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q169A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S170A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
R171A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q172A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F173A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
Q174*,A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
G184*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F267A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
W268A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D271A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L272A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G273A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
A274D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L275A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G456A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
N457A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G460A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
V462A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
T463A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

Preferred variants has one or more of the following substitutions or deletions: Q174*, D183*, G184*, K269S.

In a specific embodiment the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4 is used as the backbone for these mutations.

General mutations in variants of the invention: increased specific activity at medium temperatures

The particularly interesting amino acid substitution are those that increase the mobility around the active site of the enzyme. This is accomplished by changes that disrupt stabilizing interaction in the vicinity of the active site, *i.e.*, within preferably 10Å or 8Å or 6Å or 4Å from any of the residues constituting the active site.

Examples are mutations that reduce the size of side chains, such as

Ala to Gly,

Val to Ala or Gly,

Ile or Leu to Val, Ala, or Gly
Thr to Ser

Such mutations are expected to cause increased flexibility in the active site region either by the introduction of cavities or by the structural rearrangements that fill the space left by the mutation.

It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more Proline residues present in the part of the α-amylase variant which is modified is/are replaced with a non-Proline residue which may be any of the possible, naturally occurring non-Proline residues, and which preferably is an Alanine, Glycine, Serine, Threonine, Valine or Leucine.

Analogously, it may be preferred that one or more Cysteine residues present among the amino acid residues with which the parent α-amylase is modified is/are replaced with a non-Cysteine residue such as Serine, Alanine, Threonine, Glycine, Valine or Leucine.

Furthermore, a variant of the invention may - either as the only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 is replaced by an Asn and/or Gln, respectively. Also of interest is the replacement, in the Termamyl-like α-amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 by an Arg.

It will be understood that the present invention encompasses variants incorporating two or more of the above outlined modifications.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

<u>α-amylase variants having increased mobility around the active</u>

site:

The mobility of α-amylase variants of the invention may be increased by replacing one or more amino acid residue at one or more positions close to the substrate site. These positions are (using the SP722 α-amylase (SEQ ID NO: 2) numbering): V56, K108, D168, Q169, Q172, L201, K269, L272, L275, K446, P459.

Therefore, in an aspect the invention relates to variants being mutated in one or more of the above mentioned positions.

Preferred substitutions are one or more of the following:

V56A,G,S,T;
K108A,D,E,Q,G,H,I,L,M,N,S,T,V;
D168A,G,I,V,N,S,T;
Q169A,D,G,H,I,L,M,N,S,T,V;
Q172A,D,G,H,I,L,M,N,S,T,V;
L201A,G,I,V,S,T;
K269A,D,E,Q,G,H,I,L,M,N,S,T,V;
L272A,G,I,V,S,T;
L275A,G,I,V,S,T;
Y295A,D,E,Q,G,H,I,L,M,N,F,S,T,V;
K446A,D,E,Q,G,H,I,L,M,N,S,T,V;
P459A,G,I,L,S,T,V.

In specific embodiments of the invention the *Bacillus* strain NCIB 12512 α-amylase having the sequence shown in SEQ ID NO: 1, or *the B. stearothermophilus* α-amylase having the sequence shown in SEQ ID NO: 3, or the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4, or the *B. amyloliquefaciens* α-amylase having the sequence shown in SEQ ID NO: 5 are used as the backbone for these mutations.

As can been seen from the alignment in Figure 1 *the B. licheniformis* α-amylase and the *B. amyloliquefaciens* α-amylase have a Glutamine at position corresponding to K269 in SP722. Further, the *B. stearothermophilus* α-amylase has a Serine at position corresponding to K269 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

Furthermore, as can been seen from the alignment in Figure 1 the *B. amyloliquefaciens* α-amylase has an Alanine at position corresponding to L272 in SP722, and the *B. stearothermophilus* α-amylase has a Isoleucine at the position corresponding to L272 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

As can been seen from the alignment in Figure 1, the *Bacillus* strain 12512 α-amylase has a Isoleucine at position corresponding to L275 in SP722. Therefore for said α-amylase this substitution is not relevant.

As can been seen from the alignment in Figure 1 *the B. amyloliquefaciens* α-amylase has a Phenylalanine at position corresponding to Y295 in SP722. Further, *the B. stearothermophilus* α-amylase has an Asparagine at position corresponding to Y295 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

As can been seen from the alignment in Figure 1 *the B. licheniformis* α-amylase and the *B. amyloliquefaciens* α-amylase have a Asparagine at position corresponding to K446 in SP722. Further, the *B. stearothermophilus* α-amylase has a Histidine at position corresponding to K446 in SP722. Therefore, for said a-amylases these substitutions are not relevant.

As can been seen from the alignment in Figure 1 the *B. licheniformis* α-amylase, the *B. amyloliquefaciens* α-amylase and the *B. stearothermophilus* α-amylase have a Serine at position corresponding to P459 in SP722. Further, the *Bacillus* strain 12512 α-amylase has a Threonine at position corresponding to P459 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

Stabilization of enzymes having high activity at medium temperatures

In a further embodiment the invention relates to improving the

stability of low temperature α-amylases (e.g, *Alteromonas haloplanctis* (Feller et al., (1994), Eur. J. Biochem 222:441-447), and medium temperature α-amylases (e.g., SP722 and SP690) possessing medium temperature activity, i.e., commonly known as psychrophilic enzymes and mesophilic enzymes. The stability can for this particular enzyme class be understood either as thermostability or the stability at Calcium depletion conditions.

Typically, enzymes displaying the high activity at medium temperatures also display severe problems under conditions that stress the enzyme, such as temperature or Calcium depletion.

Consequently, the objective is to provide enzymes that at the same time display the desired high activity at medium temperatures without loosing their activity under slightly stressed conditions.

The activity of the stabilized variant measured at medium temperatures should preferably be between 100% or more and 50%, and more preferably between 100% or more and 70%, and most preferably between 100% or more and 85% of the original activity at that specific temperature before stabilization of the enzyme and the resulting enzyme should withstand longer incubation at stressed condition than the wild type enzyme.

Contemplated enzymes include α-amylases of, *e.g.*, bacterial or fungal origin.

An example of such a low temerature α-amylase is the one isolated from *Alteromonas haloplanctis* (Feller et al., (1994), Eur. J. Biochem 222:441-447). The crystal structure of this alpha-amylase has been solved (Aghajari et al., (1998), Protein Science 7:564-572).

The *A. haloplanctis* alpha-amylase (5 in alignment shown in Fig. 4) has a homology of approximately 66% to porcine pancreatic alpha-amylase (PPA) (3 in the alignment shown in Fig. 4). The PPA 3D structure is known, and can be obtained from Brookhaven database under the name 1OSE or 1DHK. Based on the homology to other more stable alpha amylases, stabilization of "the low temperature highly active enzyme" from *Alteromonas haloplanctis* alpha-amylase, can be obtained and at the same time retaining the

desired high activity at medium temperatures.

Figure 4 shown a multiple sequence alignments of five α-amylases, including the AHA and the PPA α-amylase. Specific mutations giving increased stability in *Alteromonas haloplantis* alpha-amylase:
T66P, Q69P, R155P, Q177R, A205P, A232P, L243R, V295P, S315R.

Methods for preparing α-amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α-amylase-encoding DNA sequences, methods for generating mutations at specific sites within the α-amylase-encoding sequence will be discussed.

Cloning a DNA sequence encoding an α-amylaseCloning a DNA sequence encoding an a-amylaseCloning a DNA sequence encoding an a-amylaseCloning a DNA sequence encoding an a-amylaseCloning a DNA sequence encoding an a-amylaseCloning a DNA sequence encoding an a-amylase

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labeled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones

would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g., the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Expression of α-amylase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α-amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an

autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such

sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α-amylases mentioned herein comprise a pre-region permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α-amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α-amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely

to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g., by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g., *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing an α-amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α-amylase variant of the invention.

Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The α-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial Applications

The α-amylase variants of this invention possesses valuable properties allowing for a variety of industrial applications. In particular, enzyme variants of the invention are applicable as a component in washing, dishwashing and hard-surface cleaning detergent compositions.

Numerous variants are particularly useful in the production of sweeteners and ethanol from starch, and/or for textile desizing. Conditions for conventional starch- conversion processes, including starch liquefaction and/or saccharification processes, are described in, *e.g.*, US 3,912,590 and in EP patent publications Nos. 252,730 and 63,909.

Detergent compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a

lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another α-amylase.

α-amylase variants of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of α-amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

The invention also relates to a method of providing α-amylases with 1) altered pH optimum, and/or 2) altered temperature optimum, and/or 3) improved stability, comprising the following steps:
i) identifying (a) target position(s) and/or region(s) for mutation of the α-amylase by comparing the molecular dynamics of two or more α-amylase 3D structures having substantially different pH, temperature and/or stability profiles,
ii) substituting, adding and/or deleting one or more amino acids in the identified position(s) and/or region(s).

In embodiment of the invention a medium temperature α-amylase is compared with a high temperature α-amylase. In another embodiment a low temperature α-amylase is compared with either a medium or a high temperature α-amylase.

The α-amylases compared should preferably be at least 70%, preferably 80%, up to 90%, such as up to 95%, especially 95% homologous with each other.

The α-amylases compared may be Termamyl-like α-amylases as defined above. In specific embodiment the α-amylases compared are the α-amylases shown in SEQ ID NO: 1 to SEQ ID NO: 8.

In another embodiment the stability profile of the α-amylases in question compared are the Ca^{2+} dependency profile.

MATERIALS AND METHODS

Enzymes:

SP722: (SEQ ID NO: 2, available from Novo Nordisk)

Termamyl™ (SEQ ID NO: 4, available from Novo Nordisk)

SP690: (SEQ ID NO: 1, available from Novo Nordisk)

Bacillus subtilis SHA273: see WO 95/10603

Plasmids

pJE1 contains the gene encoding a variant of SP722 α-amylase (SEQ ID NO: 2): viz. deletion of 6 nucleotides corresponding to amino acids D183-G184 in the mature protein. Transcription of the JE1 gene is directed from the *amyL* promoter. The plasmid further more contains the origin of replication and *cat*-gene conferring resistance towards kanamycin obtained from plasmid pUB110 (Gryczan, TJ et al. (1978), J. Bact. 134:318-329).

Methods:

Construction of library vector pDorK101

The *E. coli/Bacillus* shuttle vector pDorK101 (described below) can be used to introduce mutations without expression of α-amylase in *E. coli* and then be modified in such way that the α-amylase is active in Bacillus. The vector was constructed as follows: The JE1 encoding gene (SP722 with the deletion of D183-G184) was inactivated in pJE1 by gene interruption in the PstI site in the 5´coding region of the SEQ ID NO: 2: SP722 by a 1.2 kb fragment containing an *E. coli* origin of replication. This fragment was PCR amplified from the pUC19 (GenBank Accession #:X02514) using the forward primer: 5´-gacctgcagtcaggcaacta-3´ and the reverse primer: 5´-tagagtcgacctgcaggcat-3´. The PCR amplicon and the pJE1 vector were digested with PstI at 37°C for 2 hours. The pJE1 vector fragment and the PCR fragment were ligated at room temperature. for 1 hour and transformed in *E. coli* by electrotransformation. The resulting vector is designated

pDorK101.

Filter screening assays

The assay can be used to screening of Termamyl-like α-amylase variants having an improved stability at high pH compared to the parent enzyme and Termamyl-like α-amylase variants having an improved stability at high pH and medium temperatures compared to the parent enzyme depending of the screening temperature setting

High pH filter assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 µg/ml kanamycin at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with glycin-NaOH buffer, pH 8.6-10.6 and incubated at room temperature(can be altered from 10°-60°C) for 15 min. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose, 0.2% starch in glycin-NaOH buffer, pH 8.6-10.6. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours. at room temperature. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Low calcium filter assay

The *Bacillus* library are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with a relevant antibiotic, *e.g.*, kanamycin or chloramphenicol, at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with carbonate/bicarbonate buffer pH 8.5-10 and with different EDTA concentrations (0.001 mM - 100 mM). The filters are incubated at room temperature for 1 hour. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose, 0.2% starch in carbonate/bicarbonate buffer pH 8.5-10. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours. at room temperature. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Method to obtaining the regions of interest:

There are three known 3D structures of bacterial α-amylases. Two of *B. licheniformis* α-amylase, Brookhaven database 1BPL (Machius et al. (1995), J. Mol. Biol. 246, p. 545-559) and 1VJS (Song et al. (1996), Enzymes for Carbohydrate 163 Engineering (Prog. Biotechnol. V 12). These two structures are lacking an important piece of the structure from the so-called B-domain, in the area around the two Calcium ions and one Sodium ion binding sites. We have therefore used a 3D structure of an α-amylase BA2

(WO 96/23874 which are a hybrid between BAN™ (SEQ ID NO. 5) and *B. licheniformis* α-amylase (SEQ ID NO. 4). On basis of the structure a model of *B. licheniformis* alpha amylase and the SP722 α-amylase has been build.

Fermentation and purification of α-amylase variants

Fermentation and purification may be performed by methods well known in the art.

Stability determination

All stability trials are made using the same set up. The method are:

The enzyme is incubated under the relevant conditions (1-4). Samples are taken at various time points, e.g., after 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (0.1M 50mM Britton buffer pH 7.3) and the activity is measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

The activity measured before incubation (0 minutes) is used as reference (100%). The decline in percent is calculated as a function of the incubation time. The table shows the residual activity after, e.g., 30 minutes of incubation.

Specific activity determination

The specific activity is determined using the Phadebas assay (Pharmacia) as activity/mg enzyme. The manufactures instructions are followed (see also below under "Assay for α-amylase activity).

Assays for α-Amylase Activity

1. Phadebas assay

α-amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer which has been mixed

with bovine serum albumin and a buffer substance and tabletted.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM $CaCl_2$, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The α-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α-amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolyzed by the α-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the α-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure α-amylase protein) of the α-amylase in question under the given set of conditions.

2. Alternative method

α-amylase activity is determined by a method employing the PNP-G7 substrate. PNP-G7 which is a abbreviation for p-nitrophenyl-α,D-maltoheptaoside is a blocked oligosaccharide which can be cleaved by an endo-amylase. Following the cleavage, the α-Glucosidase included in the kit digest the substrate to liberate a free PNP molecule which has a yellow colour and thus can be measured by visible spectophometry at λ=405nm. (400-420 nm.). Kits containing PNP-G7 substrate and α-Glucosidase is manufactured by Boehringer-Mannheim (cat.No. 1054635).

To prepare the substrate one bottle of substrate (BM 1442309) is added to 5 ml buffer (BM1442309). To prepare the α-Glucosidase one bottle of α-Glucosidase (BM 1462309) is added to 45 ml buffer (BM1442309). The working solution is made by mixing 5 ml α-Glucosidase solution with 0.5 ml substrate.

The assay is performed by transforming 20μl enzyme solution to a 96 well microtitre plate and incubating at 25°C. 200 μl working solution, 25°C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 15 sec. over 3 minutes at OD 405 nm.

The slope of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the α-amylase in question under the given set of conditions.

General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

1. Select regions of interest for modification in the parent enzyme
2. Decide on mutation sites and non-mutated sites in the selected region
3. Decide on which kind of mutations should be carried out, e.g. with respect to the desired stability and/or performance of the variant to be constructed
4. Select structurally reasonable mutations.
5. Adjust the residues selected by step 3 with regard to step 4.
6. Analyze by use of a suitable dope algorithm the nucleotide distribution.
7. If necessary, adjust the wanted residues to genetic code realism (e.g., taking into account constraints resulting from the genetic code (e.g. in order to avoid introduction of stop codons))(the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted)
8. Make primers

9. Perform random mutagenesis by use of the primers

10. Select resulting α-amylase variants by screening for the desired improved properties.

Suitable dope algorithms for use in step 6 are well known in the art. One algorithm is described by Tomandl, D. et al., Journal of Computer-Aided Molecular Design, 11 (1997), pp. 29-38). Another algorithm, DOPE, is described in the following:

The dope program

The "DOPE" program is a computer algorithm useful to optimize the nucleotide composition of a codon triplet in such a way that it encodes an amino acid distribution which resembles most the wanted amino acid distribution. In order to assess which of the possible distributions is the most similar to the wanted amino acid distribution, a scoring function is needed. In the "Dope" program the following function was found to be suited:

$$s \equiv \prod_{i=1}^{N} \left(\frac{x_i^{y_i}}{y_i^{y_i}} \frac{(1-x_i)^{1-y_i}}{(1-y_i)^{1-y_i}} \right)^{w_i} ,$$

where the x_i's are the obtained amounts of amino acids and groups of amino acids as calculated by the program, y_i's are the wanted amounts of amino acids and groups of amino acids as defined by the user of the program (e.g. specify which of the 20 amino acids or stop codons are wanted to be introduced, e.g. with a certain percentage (e.g. 90% Ala, 3% Ile, 7% Val), and w_i's are assigned weight factors as defined by the user of the program (e.g., depending on the importance of having a specific amino acid residue inserted into the position in question). N is 21 plus the number of amino acid groups as defined by the user of the program. For purposes of this function 0^0 is defined as being 1.

A Monte-Carlo algorithm (one example being the one described by Valleau, J.P. & Whittington, S.G. (1977) A guide to Mont Carlo

for statistical mechanics: 1 Highways. In "Stastistical Mechanics, Part A" Equlibrium Techniqeues ed. B.J. Berne, New York: Plenum) is used for finding the maximum value of this function. In each iteration the following steps are performed:

1. A new random nucleotide composition is chosen for each base, where the absolute difference between the current and the new composition is smaller than or equal to *d* for each of the four nucleotides G,A,T,C in all three positions of the codon (see below for definition of *d*).
2. The scores of the new composition and the current composition are compared by the use of the function s as described above. If the new score is higher or equal to the score of the current composition, the new composition is kept and the current composition is changed to the new one. If the new score is smaller, the probability of keeping the new composition is $\exp\left(1000\left(new\_score - current\_score\right)\right)$.

A cycle normally consists of 1000 iterations as described above in which *d* is decreasing linearly from 1 to 0. One hundred or more cycles are performed in an optimization process. The nucleotide composition resulting in the highest score is finally presented.

EXAMPLES

EXAMPLE 1

Example on Homology building of Termamyl™

The overall homology of the *B. licheniformis* α-amylase (in the following referred to as Termamyl™) to other Termamyl-like α-amylases is high and the percent similarity is extremely high. The similarity calculated for Termamyl™ to BSG (the *B. stearothermophilus* α-amylase having SEQ ID NO: 3), and BAN (the *B. amyloliquefaciens* α-amylase having SEQ ID NO: 5) using the University of Wisconsin Genetics Computer Group's program GCG gave 89% and 78%, respectively. TERM has a deletion of 2 residues

between residue G180 and K181 compared to BAN™ and BSG. BSG has a deletion of 3 residues between G371 and I372 in comparison with BAN™ and Termamyl™. Further BSG has a C-terminal extension of more than 20 residues compared to BAN™ and Termamyl™. BAN™ has 2 residues less and Termamyl has one residue less in the N-terminal compared to BSG.

The structure of the *B. licheniformis* (Termamyl™) and of the *B. amyloliquefaciens* α-amylase (BAN™), respectively, was model built on the structure disclosed in Appendix 1 of WO 96/23974. The structure of other Termamyl-like α-amylases (*e.g.* those disclosed herein) may be built analogously.

In comparison with the α-amylase used for elucidating the present structure, Termamyl™ differs in that it lacks two residues around 178-182. In order to compensate for this in the model structure, the HOMOLOGY program from BIOSYM was used to substitute the residues in equivalent positions in the structure (not only structurally conserved regions) except for the deletion point. A peptide bond was established between G179(G177) and K180(K180) in Termamyl™(BAN™). The close structural relationship between the solved structure and the model structure (and thus the validity of the latter) is indicated by the presence of only very few atoms found to be too close together in the model.

To this very rough structure of Termamyl™ was then added all waters (605) and ions (4 Calcium and 1 Sodium) from the solved structure (See Appendix 1 of WO 96/23874) at the same coordinates as for said solved structure using the INSIGHT program. This could be done with only few overlaps - in other words with a very nice fit. This model structure were then minimized using 200 steps of Steepest descent and 600 steps of Conjugated gradient (see Brooks et al 1983, J. Computational Chemistry 4, p.187-217). The minimized structure was then subjected to molecular dynamics, 5ps heating followed by up to 200ps equilibration but more than 35ps. The dynamics as run with the Verlet algorithm and the e-quilibration temperature 300K were kept using the Behrendsen coupling to a water bath (Berendsen et. al., 1984, J. Chemical

Physics 81, p. 3684-3690). Rotations and translations were removed every pico second.

EXAMPLE 2

Method of extracting important regions for identifying α-amylase variants with improved pH stability and altered temperature activity

The X-ray structure and/or the model build structure of the enzyme of interest, here SP722 and Termamyl™, are subjected to molecular dynamics simulations. The molecular dynamics simulation are made using the CHARMM (from Molecular simulations (MSI)) program or other suited program like, e.g., DISCOVER (from MSI). The molecular dynamic analysis is made in vacuum, or more preferred including crystal waters, or with the enzyme embedded in water, *e.g.*, a water sphere or a water box. The simulation are run for 300 pico seconds (ps) or more, *e.g.*, 300-1200 ps. The isotropic fluctuations are extracted for the CA carbons of the structures and compared between the structures. Where the sequence has deletions and/or insertions the isotropic fluctuations from the other structure are inserted thus giving 0 as difference in isotropic fluctuation. For explanation of isotropic fluctuations see the CHARMM manual (obtainable from MSI).

The molecular dynamics simulation can be done using standard charges on the chargeable amino acids. This is Asp and Glu are negatively charged and Lys and Arg are positively charged. This condition resembles the medium pH of approximately 7. To analyze a higher or lower pH, titration of the molecule can be done to obtain the altered pKa's of the standard titrateable residues normally within pH 2-10; Lys, Arg, Asp, Glu, Tyr and His. Also Ser, Thr and Cys are titrateable but are not taking into account here. Here the altered charges due to the pH has been described as both Asp and Glu are negative at high pH, and both Arg and Lys are uncharged. This imitates a pH around 10 to 11 where the titration of Lys and Arg starts, as the normal pKa of these residues are around 9-11.

1. The approach used for extracting important regions for identifying α-amylase variants with high pH stability:

The important regions for constructing variants with improved pH stability are the regions which at the extreme pH display the highest mobility, i.e., regions having the highest isotropic fluctuations.

Such regions are identified by carrying out two molecular dynamics simulations: i) a high pH run at which the basic amino acids, Lys and Arg, are seen as neutral (i.e. not protonated) and the acidic amino acids, Asp and Glu, have the charge (-1) and ii) a neutral pH run with the basic amino acids, Lys and Arg, having the net charge of (+1) and the acidic amino acids having a charge of (-1).

The two run are compared and regions displaying the relatively higher mobility at high pH compared to neutral pH analysis were identified.

Introduction of residues improving general stability, e.g., hydrogen bonding, making the region more rigid (by mutations such as Proline substitutions or replacement of Glycine residues), or improving the charges or their interaction, improves the high pH stability of the enzyme.

2. The approach used for extracting regions for identifying α-amylase variants with increased activity at medium temperatures:

The important regions for constructing variants with increased activity at medium temperature was found as the difference between the isotropic fluctuations in SP722 and Termamyl, i.e., SP722 minus Termamyl CA isotrophic fluctuations, The regions with the highest mobility in the isotrophic fluctuations were selected. These regions and there residues were expected to increase the activity at medium temperatures. The activity of an alpha-amylase is only expressed if the correct mobility of certain residues are present. If the mobility of the residues is too low the activity is decreased or abandoned.

EXAMPLE 3

Construction, by localized random, doped mutagenesis, of Termamyl-like α-amylase variants having an improved Ca2+ stability at medium temperatures compared to the parent enzyme

To improve the stability at low calcium concentration of α-amylases random mutagenesis in pre-selected region was performed.

Region:	Residue:
SAI:	R181-W189

The DOPE software (see Materials and Methods) was used to determine spiked codons for each suggested change in the SA1 region minimizing the amount of stop codons (see table 1). The exact distribution of nucleotides was calculated in the three positions of the codon to give the suggested population of amino acid changes. The doped regions were doped specifically in the indicated positions to have a high chance of getting the desired residues, but still allow other possibilities.

Table 1:
Distribution of amino acid residues for each position
R181: 72% R, 2% N, 7% Q, 4% H, 4%K, 11%S
G182: 73% G, 13% A, 12% S, 2% T
K185: 95% K, 5% R
A186: 50% A, 4% N, 6% D, 1%E, 1% G, 1% K, 5% S, 31% T
W187: 100% W

D188: 100% D
W189: 92% W, 8% S

The resulting doped oligonucleotide strand is shown in table 2 as sense strand: with the wild type nucleotide and amino acid sequences and the distribution of nucleotides for each doped position.

Table 2:

Position	181	182	185	186	187	188	189
Amino acid seq.	Arg	Gly	Lys	Ala	Thr	Asp	Thr
Wt nuc. seq.	cga	ggt	aaa	gct	tgg	gat	tgg

Forward primer (SEQ ID NO: 15):
FSA: 5´-caa aat cgt atc tac aaa ttc 123 456 a7g 8910 tgg gat t11g gaa gta gat tcg gaa aat-3´

Distribution of nucleotides for each doped Position
1: 35% A, 65% C
2: 83% G, 17% A
3: 63% G, 37% T
4: 86% G, 14% A
5: 85% G, 15% C
6: 50% T, 50% C
7: 95% A, 5%G
8: 58% G, 37% A, 5% T
9: 86% C, 13% A, 1% G
10: 83% T, 17% G
11: 92% G, 8% C

Reverse primer (SEQ ID NO: 16):
RSA: 5´-gaa ttt gta gat acg att ttg-3'

Random mutagenesis

The spiked oligonucleotides apparent from Table 2 (which by a common term is designated FSA) and reverse primers RSA for the SA1 region and specific SEQ ID NO: 2: SP722 primers covering the SacII and the DraIII sites are used to generate PCR-library-fragments by the overlap extension method (Horton et al., Gene, 77 (1989), pp. 61-68) with an overlap of 21 base pairs. Plasmid pJE1 is template for the Polymerase Chain Reaction. The PCR fragments are cloned in the *E. coli/Bacillus* shuttle vector pDork101 (see Materials and Methods) enabling mutagenesis in *E. coli* and immediate expression in *Bacillus subtilis* preventing lethal accumulation of amylases in *E. coli*. After establishing

the cloned PCR fragments *in E. coli*, a modified pUC19 fragment is digested out of the plasmid and the promoter and the mutated Termamyl gene is physically connected and expression can take place in *Bacillus*.

Screening

The library may be screened in the low calcium filter assays described in the "Material and Methods" section above.

EXAMPLE 4

Construction of variants of amylase SEQ ID NO: 1 (SP690)

The gene encoding the amylase from SEQ ID NO: 1 is located in a plasmid pTVB106 described in WO96/23873. The amylase is expressed from the amyL promoter in this construct in *Bacillus subtilis*.

A variant of the protein is delta(T183-G184) +Y243F+Q391E+K444Q. Construction of this variant is described in WO96/23873.

Construction of delta(T183-G184) + N195F by the mega-primer method as described by Sarkar and Sommer, (1990), BioTechniques 8: 404-407.

Gene specific primer B1 (SEQ ID NO: 17) and mutagenic primer 101458 (SEQ ID NO: 19) were used to amplify by PCR an approximately 645 bp DNA fragment from a pTVB106-like plasmid (with the delta(T183-G184) mutations in the gene encoding the amylase from SEQ ID NO: 1).

The 645 bp fragment was purified from an agarose gel and used as a mega-primer together with primer Y2 (SEQ ID NO: 18) in a second PCR carried out on the same template.

The resulting approximately 1080 bp fragment was digested with restriction enzymes BstEII and AflIII and the resulting approximately 510 bp DNA fragment was purified and ligated with the pTVB106-like plasmid (with the delta(T183-G184) mutations in the gene encoding the amylase from SEQ ID NO: 1) digested with the same enzymes. Competent *Bacillus subtilis* SHA273 (amylase and protease low) cells were transformed with the ligation and

Chlorampenicol resistant transformants and was checked by DNA sequencing to verify the presence of the correct mutations on the plasmid.

primer B1: (SEQ ID NO: 17)

5' CGA TTG CTG ACG CTG TTA TTT GCG 3'

primer Y2: (SEQ ID NO: 18)

5' CTT GTT CCC TTG TCA GAA CCA ATG 3'

primer 101458 (SEQ ID NO: 19):

5' GT CAT AGT TGC CGA AAT CTG TAT CGA CTT C 3'

The construction of variant: delta(T183-G184) + K185R+A186T was carried out in a similar way except that mutagenic primer 101638 was used.

primer 101638: (SEQ ID NO: 20)

5' CC CAG TCC CAC GTA CGT CCC CTG AAT TTA TAT ATT TTG 3'

Variants: delta(T183-G184) +A186T, delta(T183-G184) +A186I, delta(T183-G184) +A186S, delta(T183-G184) +A186N are constructed by a similar method except that pTVB106-like plasmid (carrying variant delta(T183-G184) + K185R+A186T) is used as template and as the vector for the cloning purpose. The mutagenic oligonucleotide (Oligo 1) is:

5' CC CAG TCC CAG NTCTTT CCC CTG AAT TTA TAT ATT TTG 3' (SEQ ID NO: 21)

N represents a mixture of the four bases: A, C, G, and T used in the synthesis of the mutagenicoli-gonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 186 in the mature amylase.

Variant: delta(T183-G184) + K185R+A186T+N195F is constructed as follows:

PCR is carried out with primer x2 (SEQ ID NO: 22) and primer 101458 (SEQ ID NO: 19) on pTVB106-like plasmid (with mutations delta(T183-G184) + K185R+A186T). The resulting DNA fragment is used as a mega-primer together with primer Y2 (SEQ ID NO: 18) in a PCR on pTVB106-like plasmid (with mutations delta(T183-G184) + N195). The product of the second PCR is digested with restriction endonucleases Acc65I and AflIII and cloned into pTVB106 like plasmid (delta(T183-G184)+N195F) digested with the same enzymes.

primer x2: (SEQ ID NO: 22)

5' GCG TGG ACA AAG TTT GAT TTT CCT G 3'

Variant: delta(T183-G184) + K185R+A186T+N195F+Y243F+ Q391E+K444Q is constructed as follows:

PCR is carried out with primer x2 and primer 101458 on pTVB106-like plasmid (with mutations delta(T183-G184) + K185R+A186T). The resulting DNA fragment is used as a mega-primer together with primer Y2 in a PCR on pTVB106 like plasmid (with mutations delta(T183-G184) +Y243F+Q391E+K444Q). The product of the second PCR is digested with restriction endonucleases Acc65I and AflIII and cloned into pTVB106 like plasmid (delta(T183-G184) +Y243F+Q391E+K444Q) digested with the same enzymes.

Example 5

Construction of site-directed α-amylase variants in the parent SP722 α-amylase (SEQ ID NO: 2)

Construction of variants of amylase SEQ ID NO: 2 (SP722) is carried out as described below.

The gene encoding the amylase from SEQ ID NO: 2 is located in a plasmid pTVB112 described in WO 96/23873. The amylase is expressed from the amyL promoter in this construct *in Bacillus subtilis*.

Construction of delta(D183-G184) + V56I by the mega-primer method as described by Sarkar and Sommer, 1990 (BioTechniques 8: 404-407).

Gene specific primer DA03 and mutagenic primer DA07 are used to amplify by PCR an approximately 820 bp DNA fragment from a pTVB112-like plasmid (with the delta(D183-G184) mutations in the gene encoding the α-amylase shown in SEQ ID NO: 2.

The 820 bp fragment is purified from an agarose gel and used as a mega-primer together with primer DA01 in a second PCR carried out on the same template.

The resulting approximately 920 bp fragment is digested with restriction enzymes NgoM I and Aat II and the resulting approximately 170 bp DNA fragment is purified and ligated with

the pTVB112-like plasmid (with the delta(D183-G184) mutations in the gene encoding the amylase shown in SEQ ID NO: 2) digested with the same enzymes. Competent *Bacillus subtilis* SHA273 (amylase and protease low) cells are transformed with the ligation and Chlorampenicol resistant transformants are checked by DNA sequencing to verify the presence of the correct mutations on the plasmid.

primer DA01: (SEQ ID NO: 23)

5′ CCTAATGATGGGAATCACTGG 3′

primer DA03: (SEQ ID NO:24)

5′ GCATTGGATGCTTTTGAACAACCG 3′

primer DA07 (SEQ ID NO:25):

5′ CGCAAAATGATATCGGGTATGGAGCC 3′

Variants: delta(D183-G184) + K108L, delta(D183-G184) + K108Q, delta(D183-G184) + K108E, delta(D183-G184) + K108V, were constructed by the mega-primer method as described by Sarkar and Sommer ,1990 (BioTechniques 8: 404-407):

PCR is carried out with primer DA03 and mutagenesis primer DA20 on pTVB112-like plasmid (with mutations delta(D183-G184)). The resulting DNA fragment is used as a mega-primer together with primer DA01 in a PCR on pTVB112-like plasmid (with mutations delta(D183-G184)). The approximately 920 bp product of the second PCR is digested with restriction endonucleases Aat II and Mlu I and cloned into pTVB112-like plasmid (delta(D183-G184)) digested with the same enzymes.

primer DA20 (SQ ID NO:26):

5′ GTGATGAACCACSWAGGTGGAGCTGATGC 3′

S represents a mixture of the two bases: C and G used in the synthesis of the mutagenic oligonucleotide and W represents a mixture of the two bases: A and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 108 in the mature amylase.

Construction of the variants: delta(D183-G184) + D168A, delta(D183-G184) + D168I, delta(D183-G184) + D168V, delta(D183-G184) + D168T is carried out in a similar way except that

mutagenic primer DA14 is used.

primer DA14 (SEQ ID NO:27):

5′ GATGGTGTATGGRYCAATCACGACAATTCC 3′

R represents a mixture of the two bases: A and G used in the synthesis of the mutagenic oligonucleotide and Y represents a mixture of the two bases: C and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 168 in the mature amylase.

Construction of the variant: delta(D183-G184) + Q169N is carried out in a similar way except that mutagenic primer DA15 is used.

primer DA15 (SEQ ID NO:28):

5′ GGTGTATGGGATAACTCACGACAATTCC 3′

Construction of the variant: delta(D183-G184) + Q169L is carried out in a similar way except that mutagenic primer DA16 is used.

primer DA16 (SEQ ID NO:29):

5′ GGTGTATGGGATCTCTCACGACAATTCC 3′

Construction of the variant: delta(D183-G184) + Q172N is carried out in a similar way except that mutagenic primer DA17 is used.

primer DA17 (SEQ ID NO:30):

5′ GGGATCAATCACGAAATTTCCAAAATCGTATC 3′

Construction of the variant: delta(D183-G184) + Q172L is carried out in a similar way except that mutagenic primer DA18 is used.

primer DA18 (SEQ ID NO:31):

5′ GGGATCAATCACGACTCTTCCAAAATCGTATC 3′

Construction of the variant: delta(D183-G184) + L201I is carried out in a similar way except that mutagenic primer DA06 is used.

primer DA06 (SEQ ID NO:32):

5′ GGAAATTATGATTATATCATGTATGCAGATGTAG 3′

Construction of the variant: delta(D183-G184) + K269S is carried out in a similar way except that mutagenic primer DA09 is

used.

primer DA09 (SEQ ID NO:33):

5' GCTGAATTTTGGTCGAATGATTTAGGTGCC 3'

Construction of the variant: delta(D183-G184) + K269Q is carried out in a similar way except that mutagenic primer DA11 is used.

primer DA11 (SEQ ID NO:34):

5' GCTGAATTTTGGTCGAATGATTTAGGTGCC 3'

Construction of the variant: delta(D183-G184) + N270Y is carried out in a similar way except that mutagenic primer DA21 is used.

primer DA21 (SEQ ID NO:35):

5' GAATTTTGGAAGTACGATTTAGGTCGG 3'

Construction of the variants: delta(D183-G184) + L272A, delta(D183-G184) + L272I, delta(D183-G184) + L272V, delta(D183-G184) + L272T is carried out in a similar way except that mutagenic primer DA12 is used.

primer DA12 (SEQ ID NO:36):

5' GGAAAAACGATRYCGGTGCCTTGGAGAAC 3'

R represents a mixture of the two bases: A and G used in the synthesis of the mutagenic oligonucleotide and Y represents a mixture of the two bases: C and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 272 in the mature amylase.

Construction of the variants: delta(D183-G184) + L275A, delta(D183-G184) + L275I, delta(D183-G184) + L275V, delta(D183-G184) + L275T is carried out in a similar way except that mutagenic primer DA13 is used.

primer DA13 (SEQ ID NO:37):

5' GATTTAGGTGCCTRYCAGAACTATTTA 3'

R represents a mixture of the two bases: A and G used in the synthesis of the mutagenic oligonucleotide and Y represents a mixture of the two bases: C and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for

amino acid position 275 in the mature amylase.

Construction of the variant: delta(D183-G184) + Y295E is carried out in a similar way except that mutagenic primer DA08 is used.

primer DA08 (SEQ ID NO:38):

5' CCCCCTTCATGAGAATCTTTATAACG 3'

Construction of delta(D183-G184) + K446Q by the mega-primer method as described by Sarkar and Sommer,1990 (BioTechniques 8: 404-407):

Gene specific primer DA04, annealing 214-231 bp downstream relative to the STOP-codon and mutagenic primer DA10 were used to amplify by PCR an approximately 350 bp DNA fragment from a pTVB112-like plasmid (with the delta(D183-G184) mutations in the gene encoding the amylase depicted in SEQ ID NO: 2).

The resulting DNA fragment is used as a mega-primer together with primer DA05 in a PCR on pTVB112 like plasmid (with mutations delta(D183-G184)). The app. 460 bp product of the second PCR is digested with restriction endonucleases SnaB I and Not I and cloned into pTVB112 like plasmid (delta(D183-G184)) digested with the same enzymes.

primer DA04 (SEQ ID NO:39):

5' GAATCCGAACCTCATTACACATTCG 3'

primer DA05 (SEQ ID NO:40):

5' CGGATGGACTCGAGAAGGAAATACCACG 3'

primer DA10 (SEQ ID NO:41):

5' CGTAGGGCAAAATCAGGCCGGTCAAGTTTGG 3'

Construction of the variants: delta(D183-G184) + K458R is carried out in a similar way except that mutagenic primer DA22 is used.

primer DA22 (SEQ ID NO:42):

5' CATAACTGGAAATCGCCCGGGAACAGTTACG 3'

Construction of the variants: delta(D183-G184) + P459S and delta(D183-G184) + P459T is carried out in a similar way except that mutagenic primer DA19 is used.

primer DA19 (SEQ ID NO:43):

5' CTGGAAATAAAWCCGGAACAGTTACG 3'

W represents a mixture of the two bases: A and T used in the synthesis of the mutagenic oligonucleotide.
Sequencing of transformants identifies the correct codon for amino acid position 459 in the mature amylase.

Construction of the variants: delta(D183-G184) + T461P is carried out in a similar way except that mutagenic primer DA23 is used.

primer DA23 (SEQ ID NO:44):

5' GGAAATAAACCAGGACCCGTTACGATCAATGC 3'

Construction of the variant: delta(D183-G184) + K142R is carried out in a similar way except that mutagenic primer DA32 is used.

Primer DA32 (SEQ ID NO: 45):

5' GAGGCTTGGACTAGGTTTGATTTTCCAG 3'

Construction of the variant: delta(D183-G184) + K269R is carried out in a similar way except that mutagenic primer DA31 is used.

Primer DA31 (SEQ ID NO: 46):

5' GCTGAATTTTGGCGCAATGATTTAGGTGCC 3'

Example 6

Construction of site-directed α-amylase variants in the parent Termamyl α-amylase (SEQ ID NO: 4)

The amyL gene, encoding the Termamyl α-amylase is located in plasmid pDN1528 described in WO 95/10603 (Novo Nordisk). Variants with substitutions N265R and N265D, respectively, of said parent α-amylase are constructed by methods described in WO 97/41213 or by the "megaprimer" approach described above.

Mutagenic oligonucleotides are:

Primer bl1 for the N265R substitution:

5' PCC AGC GCG CCT AGG TCA CGC TGC CAA TAT TCA G (SEQ ID NO: 56)

Primer bl2 for the N265D substitution:

5' PCC AGC GCG CCT AGG TCA TCC TGC CAA TAT TCA G (SEQ ID NO: 57)

P represents a phosphate group.

Example 7

Determination of pH stability at alkaline pH of variants of the parent α-Amylase having the amino acid sequence shown in SEQ ID NO:2.

In this serie of analysis purified enzyme samples were used. The measurements were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5. The solutions were incubated at 75°C.

After incubation for 20 and 30 min the residual activity was measured using the PNP-G7 assay (described in the "Materials and Methods" section above). The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 75°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 20 min	Residual activity after 30 min
Δ(D183-G184)+M323L	56 %	44 %
Δ(D183-G184)+M323L+R181S	67 %	55 %
Δ(D183-G184)+M323L+A186T	62 %	50 %

In an other series of analysis culture supernatants were used. The measurements were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5. The solutions were incubated at 80°C.

After incubation for 30 minutes the residual activity was measured using the Phadebas assay (described in the "Materials and Method" secion above. The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding

reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 80°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 30 min
Δ(D183-G184)	4 %
Δ(D183-G184)+P459T	25 %
Δ(D183-G184)+K458R	31 %
Δ(D183-G184)+K311R	10 %

Example 8

Determination of calcium stability at alkaline pH of variants of the parent α-Amylase having the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 4.

A: Calcium stability of variants of the sequence in SEQ ID NO: 1

The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 50°C.

After incubation for 20 and 30 minutes the residual activity was measured using the PNP-G7 assay (described above). The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 50°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 1) and for the variants in question.

Variant	Residual activity after 20 min	Residual activity after 30 min

Δ(T183-G184)	32 %	19 %
Δ(T183-G184)+ A186T	36 %	23 %
Δ(T183-G184)+K185R+A186T	45 %	29 %
Δ(T183-G184)+A186I	35 %	20 %
Δ(T183-G184)+N195F	44 %	n.d.

n.d.= Not determinated

B: Calcium stability of variants of the sequence in SEQ ID NO: 2

In this serie of analysis purified samples of enzymes were used. The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 50°C.

After incubation for 20 and 30 minutes the residual activity was measured using the PNP-G7 assay (described above). The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 50°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 20 min	Residual activity after 30 min
Δ(D183-G184)+M323L	21 %	13 %
Δ(D183-G184)+M323L+R181S	32 %	19 %
Δ(D183-G184)+M323L+A186T	28 %	17 %
Δ(D183-G184)+M323L+A186R	30 %	18 %

Variant	Residual activity after 20 min	Residual activity after 30 min
Δ(D183-G184)	30%	20%

Δ(D183-G184)+N195F	55%	44%

In this serie of analysis culture supernatants were used. The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 50°C.

After incubation for 30 minutes the residual activity was measured using the Phadebas assay as described above. The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 50°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 30 min
Δ(D183-G184)	0 %
Δ(D183-G184)+P459T	19 %
Δ(D183-G184)+K458R	18 %
Δ(D183-G184)+T461P	13 %
Δ(D183-G184)+E346Q+K385R	4 %

C: Calcium stability of variants of the sequence in SEQ ID NO: 4

The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 60°C for 20 minutes.

After incubation for 20 minutes the residual activity was measured using the PNP-G7 assay (described above). The residual activity in the samples was measured using Britton Robinson

buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 60°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 4) and for the variants in question.

Variant	Residual activity after 20 min
Termamyl (SEQ ID NO: 4)	17 %
N265R	28 %
N265D	25 %

Example 9:

Activity measurement at medium temperature of α-Amylases having the amino acid sequence shown in SEQ ID NO: 1.

A: α-Amylase activity of variants of the sequence in SEQ ID NO:1

The measurement were made using solutions of the respective variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in the samples was measured at 37°C using 50 mM Britton Robinson buffer pH 7.3 and at 25°C using 50 mM CAPS buffer pH 10.5.

The temperature dependent activity and the percentage of the activity at 25°C relative to the activity at 37°C is shown in the table below for the parent enzyme (SEQ ID NO: 1) and for the variants in question.

Variant	NU/mg 25°C	NU/mg 37°C	NU(25°C) / NU(37°C)
SP690	1440	35000	4.1 %
Δ(T183-G184)	2900	40000	7.3 %
Δ(T183-G184)+K269S	1860	12000	15.5 %
Δ(Q174)	3830	38000	7.9 %

Another measurement was made using solutions of the respective variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in the samples was measured at 37°C and 50°C using 50 mM Britton Robinson buffer pH 7.3.

The temperature dependent activity and the percentage of the activity at 37°C relative to the activity at 50°C is shown in the table below for the parent enzyme (SEQ ID NO: 1) and for the variants in question.

Variant	NU/mg 37°C	NU/mg 50°C	NU(37°C) / NU(50°C)
SP690 (seq ID NO: 1)	13090	21669	60 %
K269Q	7804	10063	78 %

B: α-Amylase activity of variants of the sequence in SEQ ID NO:2

The measurement were made using solutions of the respective variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in the samples was measured at both 25°C and 37°C using 50 mM Britton Robinson buffer pH 7.3.

The temperature dependent activity and the percentage of the activity at 25°C relative to the activity at 37°C is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	NU/mg 25°C	NU/mg 37°C	NU(25°C) / NU(37°C)
Δ(D183-G184)+M323L	3049	10202	30 %
Δ(D183-G184)+M323L+R181S	18695	36436	51 %

C: α-Amylase activity of variants of the sequence in SEQ ID NO:4

The measurement were made using solutions of the respective

variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in the samples was measured at both 37°C using 50 mM Britton Robinson buffer pH 7.3 and at 60°C using 50 mM CAPS buffer pH 10.5.

The temperature dependent activity and the percentage of the activity at 37°C relative to the activity at 60°C is shown in the table below for the parent enzyme (SEQ ID NO: 4) and for the variants in question.

Variant	NU/mg 37°C	NU/mg 60°C	NU(37°C) / NU(60°C)
Termamyl	7400	4350	170 %
Q264S	10000	4650	215 %

Example 10

Construction of variants of parent hybrid BAN:1-300/Termamyl:301-483 α-amylase

Plasmid pTVB191 contains the gene encoding hybrid α-amylase BAN:1-300/Termamyl:301-483 as well as an origin of replication functional in *Bacillus subtilis* and the *cat* gene conferring chloramphenicol resistance.

Variant BM4 (F290E) was constructed using the megaprimer approach (Sarkar and Sommer, 1990) with plasmid pTVB191 as template.

Primer p1 (SEQ ID NO: 52) and mutagenic oligonucleotide bm4 (SEQ ID NO: 47) were used to amplify a 444 bp fragment with polymerase chain reaction (PCR) under standard conditions. This fragment was purified from an agarose gel and used as 'Megaprimer' in a second PCR with primer p2 (SEQ ID NO: 53) resulting in a 531 bp fragment. This fragment was digested with restriction endonucleases HinDIII and Tth111I. The 389 bp fragment produced by this was ligated into plasmid pTVB191 that had been cleaved with the same two enzymes. The resulting plasmid was transformed into *B. subtilis* SHA273. Chloramphenicol resistant clones were selected by growing the transformants on plates containing chloramphenicol as well as insoluble starch. Clones expressing an active α-amylase were isolated by selecting clones that formed halos after staining the plates with iodine vapour. The identity of the introduced mutations was confirmed by DNA sequencing.

Variants BM5(F290K), BM6(F290A), BM8(Q360E) and BM11(N102D) were constructed in a similar way. Details of their construction are given below.

Variant: BM5(F290K)
mutagenic oligonucleotide: bm5 (SEQ ID NO: 48)
Primer (1st PCR): p1 (SEQ ID NO: 52)
Size of resulting fragment: 444 bp
Primer (2nd PCR): p2 (SEQ ID NO: 53)

Restriction endonucleases: HinDIII, Tth111I
Size of cleaved fragment: 389 bp

Variant: BM6(F290A)
mutagenic oligonucleotide: bm6 (SEQ ID NO: 49)
Primer (1st PCR): p1 (SEQ ID NO: 52)
Size of resulting fragment: 444 bp
Primer (2nd PCR): p2 (SEQ ID NO: 53)
Restriction endonucleases: HinDIII, Tth111I
Size of cleaved fragment: 389 bp

Variant: BM8(Q360E)
mutagenic oligonucleotide: bm8 (SEQ ID NO: 50)
Primer (1st PCR): p1 (SEQ ID NO: 52)
Size of resulting fragment: 230 bp
Primer (2nd PCR): p2 (SEQ ID NO: 53)
Restriction endonucleases: HinDIII, Tth111I
Size of cleaved fragment: 389 bp

Variant: BM11(N102D)
mutagenic oligonucleotide: bm11 (SEQ ID NO: 51)
Primer (1st PCR): p3 (SEQ ID NO: 54)
Size of resulting fragment: 577
Primer (2nd PCR): p4 (SEQ ID NO: 55)
Restriction endonucleases: HinDIII, PvuI
Size of cleaved fragment: 576

Mutagenic oligonucleotides:
bm4 (SEQ ID NO: 47): F290E
primer 5' GTG TTT GAC GTC CCG CTT CAT GAG AAT TTA CAG G
bm5 (SEQ ID NO: 48): F290K
primer 5' GTG TTT GAC GTC CCG CTT CAT AAG AAT TTA CAG G
bm6 (SEQ ID NO: 49): F290A
primer 5' GTG TTT GAC GTC CCG CTT CAT GCC AAT TTA CAG G
bm8 (SEQ ID NO: 50): Q360E
primer 5' AGG GAA TCC GGA TAC CCT GAG GTT TTC TAC GG

bm11 (SEQ ID NO: 51): N102D

primer 5' GAT GTG GTT TTG GAT CAT AAG GCC GGC GCT GAT G

Other primers:

p1: 5' CTG TTA TTA ATG CCG CCA AAC C (SEQ ID NO: 52)

p2: 5' G GAA AAG AAA TGT TTA CGG TTG CG (SEQ ID NO: 53)

p3: 5' G AAA TGA AGC GGA ACA TCA AAC ACG (SEQ ID NO: 54)

p4: 5' GTA TGA TTT AGG AGA ATT CC (SEQ ID NO: 55)

Example 11

α-Amylase activity at alkaline pH of variants of parent BAN:1-300/Termamyl:301-483 hybrid α-amylase.

The measurements were made using solutions for the respective enzymes and utilizing the Phadebas assay (described above). The activity was measured after incubating for 15 minutes at 30°C in 50 mM Britton-Robinson buffer adjusted to the indicated pH by NaOH.

NU/mg enzyme

pH	wt	Q360E	F290A	F290K	F290E	N102D
8.0	5300	7800	8300	4200	6600	6200
9.0	1600	2700	3400	2100	1900	1900

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